ATTACHMENT C

IN THE MATTER OF
US Patent Application No. 09/380,327
by ROBERTSON et al.

Declaration under U.S.C.§ Rule 132

FIRST STATUTORY DECLARATION

I, David Alexander Clark of 444 Smith Ave., Burlington in the Province of Ontario, Canada, do solemnly and sincerely declare as follows:

1. I am the same David Alexander Clark who made a statutory declaration dated 16 December 2002 in respect of this matter.

2. I have read and understood the second office action dated 11 March 2003 issued in respect of this application. I have also read and understood the claims as presently on file, and as now proposed to be amended.

3. I note that as a result of these amendments the claims now require that

a) the antigen to which the prospective mother is exposed is either a sperm antigen, or an MHC Class I antigen of the prospective father which is present on leukocytes or in the seminal plasma of the prospective father,

b) the TGFβ is selected from the group consisting of $TGF\beta_1$, $TGF\beta_2$, $TGF\beta_3$ and activin, and

c) the method is directed to treating an infertility condition.

d) the method is directed to inducing immune tolerance.

4. The Examiner has stated at page 14 of the office action

> "In response to Applicant's argument that the novel TGF-β_2 in Clark et al is different from the conventional isoform of TGFβ, and one would not have expected that administering a conventional TGFβ isoform would prove beneficial in preventing infertility, the claims are not drawn to preventing infertility. Further, claims 75 to 76 recite TGFβ is modified comprises (sic) substitution, deletion, or addition mutants or peptide fragments of TGFβ which are clearly non-conventional TGFβ."

I understand that, while the applicants do not concede the correctness of the Examiner's objection on this point, claims 75 to 77 are to be cancelled without prejudice, and that the applicants are reserving the right to pursue these claims via a divisional application.

5. As a result of this amendment, the claims no longer encompass substitution, deletion, or addition mutants or peptide fragments of TGFβ, ie. do not encompass non-conventional TGFβ.

6. Furthermore, the Examiner has stated at page 12 of the office action:

> "Clark *et al* teach bioactive TGFβ is known to suppress the generation of cytotoxic cells in vitro and has immunosuppressive activity that leads to induction of tolerance in vivo during the first trimester pregnancy in humans".

I consider that the Examiner is completely incorrect in her interpretation of the Clark *et al.* reference, of which I am first author.

7. As discussed in Clause 26 of my previous declaration, the Clark *et al.* reference discloses the up-regulation of release *in vitro* of *non-conventional* TGFβ from $CD56^+$ cells obtained

from decidua of human first trimester pregnancy. The non-conventional TGFβ was assayed *in vitro* using mouse cytotoxic T lymphocyte (CTL) generation. This assay was chosen as a means to confirm that the non-conventional TGFβ exhibits one of the known biological activities of TGFβs.

8. However, the results from this assay alone do not indicate or suggest the biological function of *non-conventional* TGFβ ***in vivo*** and, therefore, the Clark *et al.* reference does not teach that the *non-conventional* TGFβ has immunosuppressive activity *in vivo*. In fact, the significance of the presence of the *non-conventional* TGFβ during the first trimester of pregnancy as shown by the Clark *et al.* reference was unknown; this is not surprising, as TGFβs were known to exhibit many biological functions, including cell division, cell growth inhibition, angiogenesis, cell migration, cell differentiation and immunosuppression. It was reasonable to assume that, at the time the present application was filed, the role of *non-conventional* TGFβ during the first trimester of pregnancy, as shown by the Clark *et al.* reference, could be to fulfil any one of the above–listed biological functions.

9. Infertility in humans and in mice was not ascribable in 1997 to lack of "immunological tolerance" to paternal antigen wherein maternal cytotoxic T lymphocytes were prevented from recognizing and rejecting allogeneic embryos. It was the understanding of a person skilled in the art in 1997 that embryo failure was related to natural killer (NK) cells, which lack T cell receptors for antigens such as paternal antigens, and are therefore not specific for any antigen. In 1997, non-antigen-specific NK cells were not considered to be part of the antigen-specific immune system that includes T and B lymphocytes. "Immunological tolerance" refers to an alteration of *antigen-specific* immune function, not non-specific cells such as NK cells; the CTL assay used by Clark et al was merely a convenient *in vitro* bioassay for immunosuppressive molecules such as TGFβ, which can suppress antigen-specific T cell responses *in vitro*.

10. The Examiner's interpretation that Clark et al teaches that *non-conventional* TGFβ has immunosuppressive activity *in vivo,* which leads to induction of immune tolerance *in vivo* during the first trimester pregnancy in humans, is incorrect. In fact, even if Clark *et al* did teach that TGFβ has immunosuppressive activity *in vivo*, this does not equate to or even foreshadow immune tolerance. Suppression of antigen-non-specific NK cells related to spontaneous abortion is not "immune tolerance", which is generally understood to be defined as a lack or deficit of *antigen-*

specific immune activity. This contrasts with induction of tolerance as it applies to Chaouat et al, because in this murine model, an active response to paternal antigen(s) was required to suppress NK cell activity. This is distinct from the "immune tolerance" required to accommodate pregnancy as defined in the applicant's specification.

11. The Examiner has also stated that my declaration states that "one would therefore not have expected that administering a conventional TGFβ isoform would prove beneficial in preventing infertility", whereas the claims are not drawn to *preventing* infertility. I note that claim 1 now defines "a method of treating an infertility condition in a mammalian prospective mother...".

12. I consider that the comments in clause 26 of my previous declaration are equally applicable to a method of treating an infertility condition in a mammalian prospective mother, as this is to be understood in the context of the subject specification.

13. I have reviewed the Examiner's comments regarding the remarks in my earlier declaration about the references by Feinberg and Chaouat, and see no reason to change my previous opinion. I consider that the Examiner has not fully appreciated the implications of my previous comments.

14. The Examiner has stated on page 12 of the Office Action that it would have been obvious to combine the teaching of Feinberg *et al.* and Clark *et al.* with the method of immunizing the female with paternal leukocyte antigen as taught by Chaouat *et al.* However, I consider that the Examiner is completely incorrect in her interpretation of the teachings of Chaouat in the light of the prior art. I therefore do not consider that it would be obvious to combine the teaching of Chaouat *et al.* and Clark and Feinberg and arrive at the applicant's invention.

15. Chaouat discloses immunizing a female *CBA/J* mouse with *BALB/c* leukocytes from spleen, which carried the paternal DBA/2 MHC class I antigen. Immunity elicited by administering paternal strain DBA/2 spleen leukocytes was not protective. It was not necessary to use spleen cells from male BALB/c mice; BALB/c spleen cells from female mice were effective in inducing the immunity.

16. Based on the accepted knowledge in the art at the time the application was made, including the Chaouat publication, one would not have expected that immunizing with *paternal*

leukocytes would prove beneficial in treating an infertility condition. In fact there were a number of studies published before the priority date of the present application which showed that immunizing human females with paternal antigen *per se* had *not* proven beneficial in treating an infertility condition, in contrast to the murine studies of Chaouat *et al.* Please refer to the attached documents:

> Illeni MT, Marelli G, Parazzini F, Acaia B, Bocciolone L, Bontempelli M, Faden D, Fedele L, Maffeis A, Radici E.
> Immunotherapy and recurrent abortion: a randomized clinical trial.
> Hum Reprod. 1994 Jul;9(7):1247-9. (Exhibit A)
>
> Ho HN, Gill TJ 3rd, Hsieh HJ, Jiang JJ, Lee TY, Hsieh CY.
> Immunotherapy for recurrent spontaneous abortions in a Chinese population.
> Am J Reprod Immunol 1991 Jan;25(1):10-5. (Exhibit B)
>
> Cauchi MN, Lim D, Young DE, Kloss M, Pepperell RJ
> Treatment of recurrent aborters by immunization with paternal cells--controlled trial.
> Am J Reprod Immunol 1991 Jan;25(1):16-7. (Exhibit C)

17. This is clear evidence that at the time the applicant's application was filed, there was uncertainty as to whether "immunizing with paternal antigen" to treat an infertility condition was efficacious. In fact, there is now very compelling evidence from a NIH-funded collaborative multi-centre study which shows that immunization of women with paternal lymphoid cells stored at 4°C overnight before inoculation is still able to induce an antibody response to paternal MHC (and hence expressed paternal antigen in immunogenic form), but that these stored cells *alone* did not improve pregnancy outcome in infertility. This paper was published in 1999, ie. after the date of filing of the present application. See:

> Ober C, Karrison T, Odem RR, Barnes RB, Branch DW, Stephenson MD, Baron B, Walker MA, Scott JR, Schreiber JR.
> Mononuclear-cell immunisation in prevention of recurrent miscarriages: a randomised trial.
> Lancet 1999 Jul 31;354(9176):365-9. (Exhibit D)

Thus even after the present filing date there was still uncertainty in the art on this issue.

18. I do not believe that the Chaouat reference provides any motivation to combine its teachings with those of Feinberg and Clark in order to arrive at the applicant's invention as claimed.

19. I consider that if the references by Feinberg *et al.*, Clark *et al.* and Chaouat *et al.* are properly interpreted in the light of the state of the art at the claimed priority date of the present application, a person of ordinary skill in the art would not have been motivated to combine the disclosures of these references in order to arrive at the invention as now claimed.

I declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are punishable by prison or fine or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of any application or patent thereon.

DECLARED at this day of 2003

David A. Clark

Before me:

A person empowered to witness Statutory Declarations under the laws of the Province of Ontario, Canada.

EXHIBIT A

Human Reproduction vol.9 no.7 pp.1247–1249, 1994

Immunotherapy and recurrent abortion: a randomized clinical trial

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We conducted a randomized trial comparing expectant management versus immunotherapy with paternal leukocytes to improve obstetric outcome in women with unexplained recurrent abortion. Eligible for the study were women with unexplained recurrent abortion (three or more miscarriages and no live birth), negative findings of immunological screening and no inhibition of the mixed lymphocyte culture. These women were seen for the first time between October 1988 and March 1991 in a network of obstetric departments in Northern Italy. Subjects positive for HLA-DR3 or with a partner positive for hepatitis virus B antigen were not eligible. A total of 44 women entered the study. Patients were randomly allocated to immunotherapy (22 women) or expectant management (22 women). Women allocated to immunotherapy were given 200×10^6 purified paternal lymphocytes before pregnancy. Median follow-up was 24 months (range 10–39) in the immunotherapy group and 25 months (range 11–38) in the expectant management group. Out of the 22 women randomized to immunotherapy, 16 became pregnant and the corresponding value was 14 in the expectant management group. Spontaneous abortion occurred in six out of the 16 pregnancies observed in the treated women. Among the 14 pregnancies observed in the expectant management group, two aborted and one late fetal death occurred. The cumulative proportions of women who became pregnant over 4 years were 37 and 45% in the immunotherapy and expectant management groups respectively; this difference was not significant. No adverse effect was observed in treated women.

Key words: immunotherapy/randomized trials/recurrent abortion

Introduction

Clinical observations have suggested that immunoreaction with paternal cells or cells from multiple unrelated donors, causing a maternal response and the development of cytotoxic antibodies against paternal lymphocytes, may improve the obstetric outcome in women with recurrent abortion (Scott *et al.*, 1987; Clark, 1989; Hill, 1990; Coulam and Goulam, 1992). Two randomized clinical trials reported a better, statistically significant, obstetric outcome in women with recurrent spontaneous abortion treated with immunotherapy in comparison with a placebo-treated group (Mowbray *et al.*, 1985; Clark and Daya, 1991), but two others did not confirm this (Cauchi *et al.*, 1991; Ho *et al.*, 1991).

This paper reports the result of a controlled clinical trial comparing expectant management and immunotherapy with paternal leukocytes in order to improve the obstetric outcome in women with unexplained recurrent abortion.

Material and methods

This was a randomized multicentre controlled trial. Eligible for study were 102 women with unexplained recurrent abortion (three or more miscarriages and no live birth) seen for the first time between June 1988 and March 1991 at the Outpatient Infertility Services of the 1st and 4th Obstetric and Gynaecology Departments of the University of Milan, of the University of Brescia and of the Ospedali Civili of Bergamo. All miscarriages occurred with the same partner before the 20th week of gestation. They had normal standard medical and gynaecological examination and hysterosalpingogram, luteal phase endometrial biopsy, glucose tolerance test, hormonal profile [three progesterone and prolactin assays in the luteal phase plus one thyroxine–tri-iodothyronine (T4–T3), thyroid stimulating hormone (TSH), free thyroxine index assay], and both the woman and her partner had their karyotypes determined from peripheral leukocytes. All these women underwent immunological screening including assays for complement components C3 and C4, immunoglobulin IgG, IgA and IgM, anticardiolipin antibodies, lupus-like anticoagulant, anti-nuclear, anti-extractable nuclear antigen (SSA and SSB), anti-nDNA anti-smooth muscle, anti-mitochondria, and anti-spermatozoa antibody assays. HLA typing was carried out for A, B, C, DR and DQ loci. Study of alloimmunity was made by seeking maternal lymphocytotoxic antibodies to paternal cells, and inhibition of the mixed lymphocyte reaction (MLR) between the couple by maternal serum. Mixed culture of 1×10^6 of the wife's lymphocytes as responders and 1×10^6 of the husband's irradiated (4000 Rad) lymphocytes as stimulators was performed in triplicate in medium RPMI-1640 (Techgen International S.A., Les Ulis, France), supplemented with AB serum (20%). As control, the culture was done in the wife's inactivated serum. After 6 days the cultured cells were harvested after 18 h pulsing with [^{3}H]thymidine.

Table I. Distribution of study subjects according to age, number of previous miscarriages and treatment allocation and outcome of the pregnancy

	Immunotherapy			Expectant management		
	Live birth	Abortion	Total	Live birth	Abortion	Total
Age (years)						
≤34	6	2	10	10	2	16
≥34	4	3	12	1	1	6
Miscarriages						
3	6	3	13	10	2	16
≥4	4	3	9	1	1	6

DNA synthesis was evaluated by liquid scintillation counting. The activity of mixed lymphocyte culture was evaluated according to the following formula:

cpm of culture in tested serum/cpm of culture in control serum×100%.

The presence of inhibition was defined as a value <40%.

Women eligible for the study had negative findings on immunological screening and no inhibition of the mixed lymphocyte culture. The partners of these women were screened for hepatitis virus B antigen and antibodies and human immunodeficiency virus antibodies. Subjects with positive findings for the DR3 HLA locus were not eligible (Maraldi *et al.*, 1986; Christiansen *et al.*, 1988).

Of the 102 subjects who underwent immunological screening, nine had positive findings for lupus-like anticoagulant and/or anticardiolipin antibodies, three had inhibition of the mixed lymphocyte culture, five had anti-HLA lymphocytotoxic antibodies, 17 had positive findings for the DR3 HLA locus and three had a partner with hepatitis virus B antigen. Another 21 women refused to enter the randomized study. Thus a total of 44 women were enrolled.

Patients were allocated to one of the following treatments by phone according to a computer-generated randomization list: immunotherapy (22 women) and expectant management (22 women). On the day of treatment, the buffy coat was obtained from 400 ml of anticoagulated blood of the husband. This was diluted with three parts of RPMI 1640 supplemented with antibiotic and layered onto a gradient (Lymphodex Fresenius, Bad Homburg, Germany) in Falcon tubes. These were centrifuged, and the lymphocyte layer was removed and washed three times with RPMI 1640 and then with saline solution. The cell concentration was adjusted in 3 ml of saline solution (200×10^6 cells) and injected once with 1 ml i.v., 1 ml intradermally and 1 ml s.c. to the woman, outside pregnancy. Women were then checked every 6 months for anticardiolipin antibodies till the end of follow-up (Moncayo *et al.*, 1990). The median follow-up was 24 months (range 10–39) for women allocated to immunotherapy and 25 months (range 11–38) for those allocated to expectant management. No specific instructions were given for clinical management of pregnancy, but clinicians were asked to manage the two treatment groups similarly.

Data analysis

The present analysis is based on information obtained to December 1992. The outcome of pregnancies with last menstrual period before this date was checked.

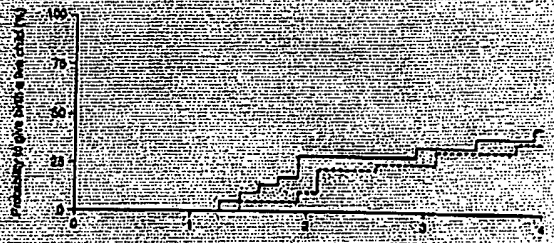

Fig. 1. Cumulative proportion of women who delivered a live-born child according to treatment allocation —, immunotherapy (22 women); – –, expectant management (22 women).

The cumulative proportion of women who gave birth to a live child was calculated by the product-limit method and the curves were compared by the log-rank test (Peto *et al.*, 1977). The event data used in computing the probability of giving birth to a live child were the date of randomization and the date of delivery. Tests of statistical significance for contingency tables were based on the usual χ^2 values, comparing observed and expected numbers of events.

Results

The distribution of study subjects according to age, history of spontaneous abortions, treatment allocation and outcome is shown in Table I. Women in the immunotherapy group tended to be older and more frequently reported four or more miscarriages than those in the expectant management group; however, these differences were not significant.

Out of the 22 women randomized to immunotherapy, 16 (73%) became pregnant, as did 14 (64%) of the 22 randomized to the expectant management group. Spontaneous abortion occurred in six of the 16 pregnancies in the former group. In the expectant management group, two women miscarried and one late fetal death occurred. Thus, women allocated to immunotherapy delivered 10 live-born children and those allocated to expectant management had 11 live-born children ($\chi^2_{adj}=0.30$; $P=$ not significant).

The cumulative proportion of women who gave birth to a live baby over a 4 year period was 37% in the immunotherapy and 45% in the expectant management group. This difference was not significant by the log-rank test (Figure 1). These estimates were consistent when the analysis was performed separately for

women with three and four or more miscarriages and in strata of age, but the number of patients >34 years and with >3 miscarriages was very small.

No adverse effect and no pathological titres of autoimmune antibodies, or anticardiolipin antibodies were observed in the treated women.

Discussion

Potential limitations of this study should be considered. We compared immunotherapy and expectant management without placebo. This choice may be questionable, since an effect of placebo may be present in our open study. However, our aim was to identify a large improvement in reproductive prognosis in the treated women, and there is no evidence that careful prenatal care greatly improves obstetric outcome. In any case, we did not find any effect of immunotherapy. Another drawback of the study is the small sample size, and hence its low statistical power. Women randomized to immunotherapy tended to be older and reported more spontaneous abortions than those randomized to expectant management. Although these differences were not significant, we cannot exclude a potentially worse prognosis in women allocated to immunotherapy since there were more older women in this group compared with the expectant management group.

With reference to other sources of bias, all randomized patients were regularly followed-up and compliance with the study protocol was complete. We observed a high percentage of women who did not become pregnant during the study period, despite active search of pregnancy. We cannot exclude unrecognized spontaneous abortion in these women, since no specific effort was made to identify early subclinical miscarriages.

In our series, the successful pregnancy rate (i.e. number of term births/number of pregnancies) was 79% in women allocated to expectant management and 65% in the immunotherapy group. These favourable rates are nearly as high (particularly for expectant management) as that in the population, but are also largely similar to the rates observed in one uncontrolled series for immunotherapy-treated women (Cauchi *et al.*, 1991) and in a series of untreated women with unexplained recurrent abortions (Parazzini *et al.*, 1988).

Finally, the dose of immunotherapy administered in the study should be considered. It has been shown that the protection effect of immunization is dose dependent, requiring for a single immunization at least 10^8 cells for optimal effects. Further, there are some suggestions that the i.v. route is the most effective, lower levels of protection being produced by the s.c. route. We administered 200×10^6 cells and only one third of the dose was given i.v. Studies reporting a favourable effect of immunotherapy used higher doses, but a lower dose was also used in studies showing favourable results with immunotherapy (Clark and Daya, 1991).

Acknowledgements

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EXHIBIT B

AMERICAN JOURNAL OF REPRODUCTIVE IMMUNOLOGY
25:10–15 © 1991 MUNKSGAARD

AJRI
Pregnancy Immunology

Immunotherapy for Recurrent Spontaneous Abortions in a Chinese Population

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ABSTRACT: *The efficacy of immunotherapy for the treatment of recurrent spontaneous abortions was tested in patients selected from the same ethnically homogeneous population of Chinese in Taiwan in whom the immunogenetics of gestational trophoblastic tumors and of recurrent spontaneous abortion had been studied. The patients, who included both primary and secondary aborters, were randomly assigned to three groups: those who were immunized with their own lymphocytes (controls) (49); those who were immunized with their husbands' lymphocytes (39); and those who were immunized with third party lymphocytes (11). The data were analyzed individually for the primary and secondary aborters and collectively for both groups combined. The number of babies born, the number of current pregnancies, and the number of recurrent abortions were not statistically significantly different between the control and the immunized groups, and a similar small number of congenital abnormalities (4–9%) occurred in both the control and immunized groups. The increase in the blocking effect for the mixed lymphocyte reaction was not related to the success of the postimmunization pregnancies. Thus, this study does not show any significant improvement in the rate of livebirths in women immunized with their husbands' lymphocytes or with third party lymphocytes compared to that in a placebo-controlled group of women.* (Am J Reprod Immunol 1991; 25:10–15).

Key Words: Primary recurrent spontaneous abortion, secondary recurrent spontaneous abortion, Immunogenetics of pregnancy in humans.

INTRODUCTION

Immunotherapy using the husband's lymphocytes or those of third party donors has been proposed as a treatment for recurrent spontaneous abortion. The efficacy of this treatment has been controversial, with some studies reporting beneficial effects generally[1–4] or in selected populations[5,6] and others reporting that psychological support alone could achieve similar successful results.[8] We undertook to explore this question in the same ethnically homogeneous Chinese population in Taiwan in whom we had previously studied the immunogenetics of gestational trophoblastic tumors[9] and of recurrent spontaneous abortions[10] using the immunization protocol of a study that had reported a beneficial effect of immunotherapy for the treatment of recurrent spontaneous abortion.[2]

Please see related editorial on page 18

In our previous study of recurrent spontaneous aborters,[10] we showed that there was an excess of HLA sharing in both primary and secondary aborters. The primary aborters shared HLA-A, DQ, and three or more of the HLA-A, B, DR, or DQ antigens. The secondary aborters did not have an excess of sharing at any one locus, but they did share three or more of the HLA-A, B, DR, or DQ antigens. There was no excess of antipaternal cytotoxic antibodies in either group of aborters, and the primary aborters had a lower level of mixed lymphocyte reaction (MLR) blocking factor than did normal couples or secondary aborters.

Based on this immunogenetic study of recurrent spontaneous aborters, we undertook an investigation of the effects of immunizing primary and secondary aborters with lymphocytes from their husbands or with lymphocytes from third party donors, and compared the effects to those of immunizing the patients with their own lymphocytes. The results of this controlled study of immunotherapy for recurrent spontaneous abortion showed that there was no beneficial effect of immunization on the outcome of pregnancy in this population; no difference in the response to immunization with the husbands' lymphocytes or with third party lymphocytes; no significant differences in the effects of immunization in either the primary or the secondary aborters; and no excess congenital abnormalities in the offspring of the immunized mothers.

MATERIALS AND METHODS

Patient Population

The patients who received immunotherapy were selected from the population in which the immunogenetics of gestational trophoblastic tumors and of recurrent spontaneous abortions had been studied,[9,10] and they were randomly assigned to the control group and to the immunotherapy groups. Both primary and secondary aborters were studied. All of the women had had three or more consecutive spontaneous abortions with the same husband.

Evaluation of the Patient Population

All of the patients and their husbands were karyotyped in order to rule out the possibility that chromosomal abnormalities were the cause of the recurrent pregnancy losses. The women underwent hysterosalpingography and hysteroscopy in order to detect any anatomic defects in the reproductive tract. The serum levels of prolactin,

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testosterone, T_3, T_4, and TSH; and the plasma glucose levels before and after meals were measured. Basal body temperature was recorded for at least three months, and midluteal phase progesterone levels were examined twice to exclude luteal-phase insufficiency. Only women whose tests were within normal limits were included in this study.

In order to screen for possible autoimmune disorders in these patients, the serum levels of immunoglobuling, the C3 and C4 components of complement, antinuclear antibody, anti-ENA (RoSSA, RoSSB, SM, RNP and scl-70), anti-single-stranded DNA, and anti-double-stranded DNA were measured. The possible presence of lupus anticoagulant was examined by assaying the prolongation of activated partial thromboplastin time and its ability to be corrected by the addition of normal plasma at a 1:1 ratio.[11] Antiphospholipid (cardiolipin and phosphotidylserine) antibody was assayed by the ELIZA method, and a titer higher than the mean plus three standard deviations of the value for 200 normal women who were not pregnant was considered to be positive.[12] Only women in whom all of these immunological parameters were within normal limits were included in this study. The husbands of these women all had normal semen analyses.

TABLE I. Patients Who Were Immunized With Their Own Lymphocytes

Patient	Age (yrs)	Obstetric Hx: Live birth	Obstetric Hx: Spontaneous abortion	HLA-sharing: DQ	DR	B	C	A	Preimmunization: Blocking effect[a]	Preimmunization: Cytotoxic antibody[b]	Postimmunization: Blocking effect[a]	Postimmunization: Cytotoxic antibody[b]	Pregnancy outcome
PA2	29	0	4	+	+	−	−	−	−	−	+	−	Female (G6PD deficiency)
PA6	31	0	4	+	+	−	+	+	−	−	−	−	Repeated abortion, 46XX
PA18	23	0	5	−	−	+	+	+	−	−	−	−	Repeated abortion, 46XY
PA11	29	0	4	+	−	−	−	−	+	−			Male
PA23	32	0	4	+	−	−	−	−	+	+	+	+	Female
PA24	25	0	4	−	+	−	−	+	−	−	−	−	Female
PA3	28	0	4	+	+	−	−	+	−	−	+	−	Male
PA66	27	0	3	+	−	−	+	+	−	−			Male
PA40	29	0	4	+	−	−	−	−	−	−	−	−	Female
PA73	30	0	3	+	+	−	−	+	−	−			Repeated abortion
PA46	30	0	3	−	−	+	+	+	−	−	−	−	Repeated abortion
PA88	28	0	3	+	+	−	+	+	−	−			Repeated abortion, 46XY
PA41	27	0	3	+	−	−	−	+	−	−			Repeated abortion, 46XX
PA10	28	0	6	+	−	−	−	+	−	−	−	−	Male
PA8	26	0	6	+	−	−	−	+	−	−	−	−	Female
PA9	29	0	3	+	−	−	+	+	−	−			Repeated abortion, 46XY
PA87	29	0	3	+	−	−	+	+	−	−			Repeated abortion, 46XX
PA67	27	0	3	+	+	−	−	+	−	−	−	−	Female
PA7	28	0	3	+	+	−	−	+	+	−	+	−	Male
PA26	28	0	4	+	−	−	−	+	−	−	−	−	Repeated abortion, 46XX
PA25	27	0	3	+	−	−	−	+	−	−			Male
PA21	36	0	6	+	+	+	−	−	+	−	+	−	Female
PA22	24	0	3	+	+	−	+	+	−	−	−	−	Repeated abortion
PA35	27	0	6	+	+	−	−	−	−	−	−	−	Male
PA99	32	0	3	+	−	−	−	+	+	−			Repeated abortion, 46XX
PA32	30	0	6	+	+	−	−	+	+	−	+	−	Pregnancy 8M
PA33	26	0	3	+	+	−	−	+	+	−	+	−	Repeated abortion, 46XX
PA98	35	0	3	+	+	−	−	−	−	−	−	−	Pregnancy 5M
PA100	30	0	4	+	−	−	−	+	−	−			Pregnancy 7M
PA113	29	0	3	+	−	−	−	+	−	−	+	−	Pregnancy 8M
PA115	29	0	3	+	−	−	−	+	−	−	−	−	Pregnancy 8M
PA117	26	0	3	+	−	−	−	+	−	−	−	−	Pregnancy 8M
PA118	28	0	3	+	−	+	−	+	−	−	+	−	Pregnancy 7M
PA119	22	0	3	+	+	−	−	+	−	−	−	−	Pregnancy 6M
PA120	29	0	3	−	−	−	−	+	−	−	−	−	Pregnancy 6M
PA121	27	0	3	+	+	−	−	+	−	+			Repeated abortion
PA122	26	0	3	+	−	−	−	+	−	−	−	−	Pregnancy 5M
SA3	29	1	4	+	−	−	+	+	−	−	+	−	Repeated abortion, 46XY
SA5	31	1	4	+	−	−	−	+	−	−	+	−	Male
SA12	24	1	3	+	+	+	+	+	−	−	+	−	Female with IUGR, VSD and PDA
SA14	26	1	3	+	−	−	+	+	−	−	+	−	Female
SA15	28	1	3	−	−	+	−	+	−	−	+	−	Female
SA28	36	2	7	+	+	+	−	−	−	−	+	−	Male
SA32	41	1	4	+	+	+	+	−	+	−	+	+	Female
SA26	25	1	3	+	+	−	+	+	−	+	+	−	Female with IUGR
SA10	26	1	5	−	−	−	+	+	+	−			Male
SA7	32	1	3	−	−	−	−	+	−	−	−	−	Repeated abortion
SA31	29	1	3	+	−	−	−	+	−	−	−	−	Repeated abortion
SA34	32	1	3	+	+	−	−	+	−	+	+	+	Female with IUGR
													Repeated abortion

[a] "+" = Sharing at the locus, "−" = no sharing.
"+" = Blocking effect >20%, "−" = blocking effect <20%.
[b] "+" = Positive antipaternal cytotoxic antibody, "−" = negative antipaternal cytotoxic antibody.
G6PD deficiency = Glucose-6-phosphate dehydrogenase deficiency, IUGR = Intrauterine growth retardation (below 10%), VSD = Ventricular septal defect, PDA = Patent ductus arteriosis.

Immunogenetic Assay Methods

The methods used for HLA typing, blocking of (MLR) and detection of complement-dependent antipaternal lymphocytotoxic antibodies have been described.[10]

Immunization Schedule

The immunization schedule was modified from Mowbray et al.[2] The control group received their own lymphocytes, and the treated groups received lymphocytes either from their husbands or from third party donors. The latter were healthy males. They were selected for donation when the husband of the patient was HBsAg-positive. The lymphocytes were separated from 120 ml of heparinized peripheral blood by Ficoll-Hypaque centrifugation under sterile conditions, washed three times with phosphate-buffered NaCl, pH 7.4 (PBS), and resuspended in 2 ml of PBS, which then contained $100–200 \times 10^8$ cells. The cell suspension was injected intradermally into several sites on the lateral aspect of both upper arms. Women who did not seroconvert after this course of immunization were given a further dose of lymphocytes prepared from 50 ml of blood. After immunization, all patients were encouraged to get pregnant. If a patient could not conceive within six months, she was reexamined, and, if she did not have any antibodies against the immunizing lymphocytes, she was reimmunized.

The study population included 49 women immunized with their own lymphocytes (controls) of whom 37 were primary aborters and 12 secondary aborters; 39 women immunized with their husbands' lymphocytes of whom 30 were primary aborters and nine secondary aborters, and 11 women immunized with lymphocytes from third party donors of whom eight were primary aborters and three secondary aborters.

The MLR blocking effect and the cytotoxic antibodies were determined periodically during pregnancy. Amniocentesis was recommended for all patients, and chromosomal analyses of many of the abortuses were performed. All of the babies delivered were registered in the study, examined carefully and followed closely by pediatricians.

TABLE II. Patients Who Were Immunized With Their Husbands' Lymphocytes

Patient	Age (yr)	Obstetric Hx: Live birth	Spontaneous abortion	HLA sharing[a]: DQ	DR	B	C	A	Preimmunization: Blocking[b] effect	Cytotoxic[c] antibody	Postimmunization: Blocking[b] effect	Cytotoxic[c] antibody	Pregnancy outcome
PA5	30	0	4	–	–	–	–	+	–	–	+	–	Male
PA6	32	0	6	+	+	–	–	+	–	–	+	–	Male
PA12	30	0	4	–	–	+	+	–	–	–	+	–	Repeated abortion, 46XY
PA14	34	0	4	+	+	–	–	+	–	–	+	–	Male
PA15	29	0	4	+	+	–	–	+	–	–	+	–	Female
PA18	34	0	5	–	–	+	+	–	+	–	+	–	Female with IUGR, PDA, and hemangioma
PA13	32	0	6	–	–	+	+	+	–	–	+	–	Repeated abortion, 46XX
PA29	26	0	3	–	+	–	–	+	–	–	+	–	Female
PA28	26	0	3	–	+	+	–	+	–	–	+	–	Male
PA36	27	0	3	+	–	–	+	+	–	–	+	–	Repeated abortion, 46XY
PA1	27	0	3	–	–	+	+	–	–	–	+	–	Female
PA72	25	0	3	+	+	+	–	+	–	–	+	–	Male
PA80	29	0	3	–	+	+	–	+	–	–	+	–	Repeated abortion, 46XX
PA41	27	0	7	+	–	–	+	+	–	–	+	–	Female
PA68	29	0	4	+	–	–	+	+	–	–	+	–	Male
PA74	27	0	3	+	+	–	–	+	–	–	+	–	Repeated abortion, 46XX
PA96	24	0	3	+	–	–	+	+	–	–	+	–	Male
PA93	23	0	6	+	–	–	+	+	–	–	+	–	Repeated abortion, 46XX
PA89	25	0	3	–	+	–	+	+	–	–	+	–	Repeated abortion, 46XY
PA22	24	0	4	–	+	–	+	–	–	–	+	–	Male
PA38	28	0	4	+	–	–	+	–	–	–	+	–	Male
PA88	28	0	3	+	–	–	+	+	–	–	+	–	Repeated abortion
PA63	25	0	4	–	+	–	+	–	–	–	+	–	IUFD at 16 wks, 46XY
PA73	30	0	3	+	–	+	–	+	–	–	+	–	Female
PA39	28	0	4	+	+	–	–	+	–	–	+	–	Female
PA101	32	0	3	+	+	–	–	+	–	–	+	–	Pregnancy 8M
PA53	23	0	6	+	+	–	–	+	–	–	+	–	Pregnancy 7M
PA79	37	0	3	+	+	+	–	+	–	–	+	–	Pregnancy 8M
PA91	30	0	3	–	–	+	+	–	–	–	+	–	Pregnancy 8M
PA90	28	0	3	+	+	–	–	+	–	–	+	–	Pregnancy 5M
PA134	30	0	3	+	+	–	–	+	–	–	+	–	Pregnancy 5M
PA136	29	0	4	+	+	–	–	+	–	–	+	–	Pregnancy 6M
SA4	28	1	3	+	–	+	+	–	–	–	+	–	Pregnancy 5M (twins)
SA21	26	2	3	+	+	–	+	+	–	–	+	–	Pregnancy 6M
SA25	28	1	3	+	+	+	–	+	–	–	+	–	Pregnancy 6M
SA9	31	1	3	+	+	+	+	+	–	–	+	–	Male
SA19	26	1	3	–	+	+	+	–	–	–	+	–	Male
SA29	27	1	4	–	+	+	+	–	–	–	+	–	Male
SA8	34	1	3	+	+	–	–	+	–	–	+	–	Female
SA11	33	1	4	–	–	+	+	+	–	–	+	–	Female
SA36	37	1	3	+	+	+	+	+	–	–	+	–	Female
												+	Male
												–	Repeated abortion

[a] "+" = Sharing at this locus, "–" = no sharing at that locus.
[b] "+" = Blocking effect >20%, "–" = blocking effect <20%.
[c] "+" = Positive antipaternal cytotoxic antibody, "–" = negative antipaternal cytotoxic antibody.
IUGR = Intrauterine growth retardation; IUFD = Intrauterine fetal death; PDA = Patent ductus arteriosus.

TABLE III. Patients Who Were Immunized With Third Party Donor Lymphocytes[a]

Patient	Age (yrs)	Obstetric Hx: Live birth	Obstetric Hx: Spontaneous abortion	HLA sharing[b]: DQ	DR	B	C	A	Preimmunization: Blocking[c] effect	Preimmunization: Cytotoxic[d] antibody	Postimmunization: Blocking[c] effect	Postimmunization: Cytotoxic[d] antibody	Pregnancy outcome
PA17	30	0	3	+	+	+	–	+	–	–	+	+	Male
PA18	32	0	3	+	–	+	–	–	–	–	+	–	Female twins
PA19	24	0	6	–	–	+	+	+	–	–	+	–	Male
PA36	28	0	4	–	–	–	+	+	–	–	–	–	Female
PA20	32	0	3	+	–	–	–	–	+	–	+	–	Male
PA82	26	0	3	+	–	–	+	–	–	–	+	–	Pregnancy 7M
PA74	27	0	4	+	–	–	+	+	?	–	–	–	Repeated abortion
PA80	31	0	3	+	–	–	+	+	+	–	+	–	Pregnancy 6M
SA1	31	1	4	–	–	–	–	+	+	–	+	–	Ectopic pregnancy
SA10	27	1	5	–	–	–	–	+	–	–	–	–	Repeated abortion, 46XX
SA18	30	1	4	+	–	+	–	+	–	–	+	+	Pregnancy 9M

[a] "+" = Sharing at that locus; "–" = no sharing at that locus.
[b] "+" = Blocking effect > 20%; "–" = blocking effect < 20%.
[c] "+" = Positive anti-paternal cytotoxic antibody; "–" = negative anti-paternal cytotoxic antibody.

The examination upon delivery included a physical examination and measurement on cord blood of the serum levels of immunoglobulin and complement, of the number of lymphocytes and their subsets and of the proliferative responses by the cord blood lymphocytes to mitogens and to allogeneic lymphocytes.

All comparisons were performed by the chi square test using a 2 × 2 contingency table, and $P < 0.05$ was taken as the level of significance.

RESULTS

The data for the patients who were immunized with their own lymphocytes (controls) are shown in Table I; for those who were immunized with their husbands' lymphocytes, in Table II; and for those who were immunized with lymphocytes from third party donors, in Table III. The analyses of these data are summarized in Tables IV (primary aborters), V (secondary aborters), and VI (combined primary and secondary aborters).

The average ages and the average and median numbers of abortions in the control and study groups were not significantly different. The extent of HLA sharing was the same in both the control and study groups, and in both cases it was greater than that found in normally fertile couples in the same population.[10] The immune responses of women immunized with their husbands' lymphocytes were not significantly different from those of women immunized with third party lymphocytes.

Twenty-three babies from 49 women were delivered in the control group, and nine women were still pregnant (> five months) when this report was written; and 26 babies from 50 women were delivered by the study groups, and 13 women were still pregnant (> five months) (Table VI). These data show that there are no significant differences in the percentage of livebirths or of total pregnancies (livebirths plus current pregnancies) between the control and study groups. The same conclusion is reached when the data are analyzed separately for the primary

TABLE IV. Summary of the Results of Treating Primary Recurrent Spontaneous Aborters With Immunotherapy

	Control group: Patient's lymphocytes		Study group: Husband's lymphocytes		Study group: Third party lymphocytes		Total	
Age (years)								
Mean	28.2 ± 2.8[a]		28.4 ± 2.6		28.8 ± 2.5		28.5 ± 2.6	
Median	28		28		29		28	
No. of abortions	3.6 ± 0.4[a]		3.9 ± 0.9		4.2 ± 1.4		4.0 ± 1.1	
	No.	%	No.		No.		No.	%
≥ three HLA antigens shared	17/37	46	16/30		3/8		18/38	47
Blocking effect > 20%								
preimmunization	6/33	18	8/29		2/8		11/37	30
postimmunization	10/33	30	23/29		5/8		28/37	76[b]
Antipaternal cytotoxic antibody								
preimmunization	1/33	3	2/29		0/8		2/37	5
postimmunization	1/33	3	7/29		1/8		8/37	22[b]
Babies delivered	14/37	38	13/30		5/8[c]		18/38	47
Current pregnancies	9/37	24	10/30		2/8		12/38	32
Repeated abortion	14/37	38	7/30		1/8		8/38	21
Abnormalities								
IUGR	0/14	0	1/13		0/6[c]		1/19	5
congenital	1/14	7	1/13		0/6[c]		1/19	5

[a] Mean ± SD.
[b] One pregnancy had twins (scored as one pregnancy).
[c] Significantly different at $P < 0.001$.
[d] Significantly different at $P < 0.05$.

TABLE V. Summary of the Results of Treating Secondary Recurrent Spontaneous Aborters With Immunotherapy

	Control group	Study group		
	Patient's lymphocytes	Husband's lymphocytes	Third party lymphocytes	Total
Age (years)				
Mean	30.0 ± 3.5*	30.0 ± 4.3	29.3 ± 1.6	29.8 ± 2.9
Median	29	28	30	29
No. of abortions	3.7 ± 0.8*	3.2 ± 0.3	4.3 ± 0.4	3.5 ± 0.4
	No. %	No.	No.	No. %
≥ three HLA antigens shared	8/12 67	6/9	0/3	6/12 42
Blocking effect ≥ 20%				
preimmunization	6/11 54	3/7	1/2	4/9 44
postimmunization	8/11 73	5/7	2/2	7/9 78
Antipaternal cytotoxic antibody				
preimmunization	3/11 27	3/7	0/2	3/9 33
postimmunization	2/11 18	3/7	1/2	4/9 44
Babies delivered	9/12 75	6/9	0/3	6/12 67
Current pregnancies	0/12 0	0/9	1/3	1/12 8
Repeated abortion	3/12 25	1/9	1/3	2/12 17
Abnormalities				
IUGR	3/9 33	0/9		0/9 0
congenital	1/9 11	0/9		0/9 0

*Mean ± SD.

aborters (Table IV) and for the secondary aborters (Table V). There was also no difference between the control and study groups when the data for couples sharing three or more HLA antigens and for couples sharing less than three HLA antigens were analyzed separately. A similar small number of congenital abnormalities was found in both the control (2/23) and the study (1/28) groups. These data show that, from the obstetric point of view, immunization of recurrent spontaneous aborters with lymphocytes from their husbands or from third party donors did not influence the outcome of pregnancy.

The serum blocking effect increased after immunization in the study group ($P < 0.001$) but not in the control group (Table VI); hence, the immunization protocol was effective. There was, however, no statistically significant relationship between a successful pregnancy and an increase in the postimmunization blocking effect. The antidonor cytotoxic antibody response increased in the primary aborters but not in the secondary aborters or in the combined groups.

DISCUSSION

The estimated prevalence of recurrent spontaneous abortions in those couples in whom the woman has had three previous spontaneous abortions has been reported to be 25–50% in both retrospective[13–15] and prospective[16–18] studies. Ten of 18 women who had unexplained recurrent spontaneous abortions whom we evaluated in our previous immunogenetic studies[9,10] had successful pregnancies without any treatment (45% prevalence of

TABLE VI. Summary of the Combined Results of Treating Primary and Secondary Recurrent Spontaneous Aborters With Immunotherapy

	Control group	Study group		
	Patient's lymphocytes	Husband's lymphocytes	Third party lymphocytes	Total
Age (years)				
Mean	28.7 ± 2.2*	28.8 ± 2.8	28.9 ± 2.2	28.8 ± 2.6
Median	28	28	30	28
No. of abortions	3.8 ± 0.7*	3.7 ± 0.8	4.2 ± 1.1	3.8 ± 1.1
	No. %	No.	No.	No. %
≥ three HLA antigens shared	25/49 51	20/39	3/11	23/50 46
Blocking effect ≥ 20%				
preimmunization	12/44 27	12/36	3/10	15/46 33
postimmunization	18/44 41	28/36	8/10	36/46 78†
Antipaternal cytotoxic antibody				
preimmunization	4/44 9	5/38	0/10	5/48 11
postimmunization	3/44 7	10/36	2/10	12/46 26
Babies delivered	23/49 47	21/39	5/11	26/50 52
Current pregnancies	9/49 18	10/39	3/11	13/50 26
Repeated abortion	17/49 35	6/39	2/11	10/50 20
Abnormalities				
IUGR	3/23 13	1/22	0/6	1/28 4
congenital	2/23 9	1/22	0/6	1/28 4

*Mean ± SD.
†Significantly different at $P < 0.001$.

recurrent spontaneous abortion) (Ho, unpublished data). This observation is consistent with the reports in the literature and with the results of the control group in the present study.

Although the results of immunotherapy for recurrent spontaneous abortion have been controversial, we believe that the weight of evidence—including the study reported here—indicates that it is not a generally useful approach to the treatment of this disease. There may be specific circumstances in which immunotherapy could be useful, but they remain to be clarified. Also, psychological factors may influence the outcome of pregnancy,[8] including those involved in evaluating immunotherapy for recurrent spontaneous abortion, but they have not been explored in adequate detail.

Changes in the serum blocking effect or in antidonor lymphocytotoxic antibodies do not provide a clear guide to the outcome of postimmunization pregnancies. Studies in experimental animals[19] and in humans[11] strongly suggest that these antibodies are the result of pregnancy or of pregnancy loss and not a determinant of it. Also, embryo transfer studies in rats[19] showed that when the appropriate combinations of embryo donor and recipient are used, the classical allele-specific class I transplantation antigen (the A[a] antigen) and the class I antigen carrying broadly shared antigenic determinants (the Pa antigen) are expressed on the surface of the embryo. The antibody response to these antigens does not harm the fetus, at least in the first pregnancy. The evidence so far, both in experimental animals and in humans, supports the hypothesis that the antibody response to the embryo is not pathogenic, at least in most circumstances, and that the majority of unexplained recurrent pregnancy losses have a genetic basis—either chromosomal abnormalities or homozygosity for recessive lethal genes. The paradigm for the latter type of genetic defect is the MHC-linked genes affecting development and susceptibility to cancer that have been demonstrated in experimental animals (20, 21) and postulated on the basis of HLA sharing in couples experiencing recurrent spontaneous abortion to exist in humans.[9,10,22]

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NOTE ADDED IN PROOF

All of the women listed as "Current pregnancies" in Tables IV and V delivered liveborn children. Two of the children of immunized primary aborters (one immunized with her husband's lymphocytes, and the other with third party lymphocytes) had IUGR; there were no other developmental abnormalities. Reanalysis of the data still showed no difference in the number of liveborn between controls (women immunized with their own lymphocytes) and women immunized with lymphocytes from their husbands or from third party donors (all χ^2 values > 0.1).

EXHIBIT C

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Treatment of Recurrent Aborters by Immunization With Paternal Cells—Controlled Trial

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ABSTRACT: A paired sequential trial was undertaken to establish whether paternal mononuclear cells improved the prognosis in couples with recurrent abortions. For this purpose, 10^7–10^8 cells obtained from the blood of partners were injected intravenously, subcutaneously, and intra-dermally into women who had had three or more consecutive miscarriages with the same partner. Control women were given normal saline injected in the same manner. The result of the sequential analysis showed that there was no significant beneficial effect of the cells compared to control. The overall success rate was 70% (32/46 couples). The success rate in patients given cells was 62% (13/21), while in those given saline it was 76% (19/25). While the overall success rate in this study compares with a number of other studies, we find an equally high success rate with non-immunized patients. We conclude that the value of immunization for the prevention of recurrent miscarriage has not been established. (Am J Reprod Immunol 25:16–17.)

INTRODUCTION

An immunological basis for recurrent miscarriage has been postulated by a number of workers. Data from experimental animals suggested that immunization with lymphocytes could lead to an improved prognosis.[1] Immunization with either paternal[2–4] or unrelated third party mononuclear cells[5] has been utilized by several workers, with success rates varying from 70–80%.

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The significance of these results however is confounded by the fact that there have been only a limited number of controlled trials to show the significance of immunotherapy. Moreover treatments based on a non-immunological rationale seem to have an equally successful outcome.[6,7]

Mowbray et al.[2] were the first to show, in a paired sequential double blind trial, that immunization with paternal cells was significantly superior to immunization with maternal cells, with success rate of 77% and 37% respectively. Carp et al.[4] in a "matched controlled trial" showed that there was a significantly higher success rate in the treated group (71.9%) compared to non-immunized controlled group (34.3%). In a "quasi-randomized" study, Cowchock et al.[3] found a success rate of 58% in immunized compared to 39% in non-immunized patients. We report here the result of a paired sequential double blind trial to determine whether paternal cells were more effective than normal physiological saline in preventing miscarriage.

MATERIALS AND METHODS

Patients were included in this study if they had three or more consecutive first trimester miscarriages with the same partner and after exclusion of other known causes of recurrent miscarriage. Assessment included full physical gynecological examination for evidence of fibroids, excluding other abnormalities of the uterus by hysterosalpingogram and/or hysteroscopy, endocrinological tests including thyroid function tests, hemoglobin A1C, and chromosomal abnormalities. Standard coagulation tests (including APTT, KCT), auto-antibody tests (including anti-nuclear antibody, anti-DNA antibody) as well as anti-cardiolipin antibody were performed, and patients were excluded if the results were abnormal. Patients were also excluded from this study if they were rhesus negative or had anti-paternal cytotoxic antibodies. Patients who had a previous normal pregnancy (secondary aborters) were not excluded from this study.

Immunization Procedures

Mononuclear cells (10^7–10^8) were prepared from 100–150 ml of heparinized blood taken from the partner, separated by density centrifugation and concentrated to 2 ml, half of which was injected I.V. and the rest injected into multiple sites intra-dermally and subcutaneously (Cauchi et al.).[5] Control patients were given 2 ml of normal saline, injected in the same manner.

Sequential Trial Methodology

A sequential trial chart was constructed before the trial from values in Armitage,[8] based on the assumption that the probability of a successful pregnancy when the husband's cells are injected was 0.85, compared to a probability of 0.50 in controls ($\theta_1 = 0.85$). The two-sided overall significance level was $\theta = 0.05$. The null hypothesis was that $\Theta = 0.50$. The maximum number of preference pairs needed to complete the trial was estimated at 27. The average sample number (ASN) was 13.4, and the expected number of pairs (N) needed to complete the trial was 27.

Randomization of Patients

Patients were allotted to one or the other treatment group using a computer generated list of numbers. Neither the treating obstetrician nor the patient knew whether they were injected with cells or saline. There was no significant difference between the two groups in relation to age, proportion of primary aborters, length of abortion history, or number of abortions per year.

A successful pregnancy was one which was continuing normally at or beyond 20 weeks gestation.

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RESULTS

Figure 1 shows the results of the paired sequential trial. No significant difference was seen between immunization with paternal cells and injection with normal saline.

The overall success rate of treatment was 70% (32/46). The success rate in patients given cells was 62% (13/21), while in those given saline was 76% (19/25). This difference is not statistically significant ($\chi^2 = 1.07$).

DISCUSSION

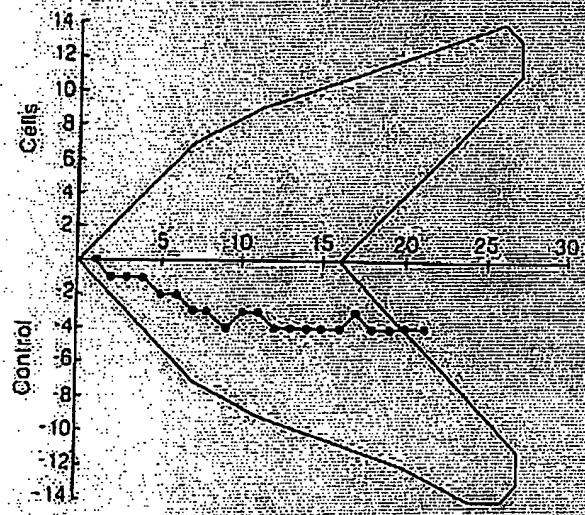
The discrepancy between our results and those claiming a significant beneficial effect of immunization with partner's lymphocytes needs to be explained. It is most likely due to the very low success rate in "control patients" in the other studies. The degree of significance obtained in the study by Moybray et al.[2] was largely dependent on the very low success rate in women given maternal cells (37%). Carp et al.[4] found that in a non-immunized control group, the success rate was 37% in those with no anti-paternal cell cytotoxic antibodies, and 50% in those with anti-paternal cell antibody (APCA) positivity. Likewise, Cowchock et al.[3] found a success rate of 39% in the untreated group. On the other hand other workers have reported success rates of up to 60% in untreated patients[5] or in patients treated by non-immunological techniques.[6]

In the only other controlled study of lymphocyte immunotherapy for recurrent spontaneous abortion,[19] there was no statistical difference between those immunized with husband's or third parties' lymphocytes compared to those given their own lymphocytes. They suggested that lymphocyte immunotherapy for recurrent spontaneous abortion might be a placebo effect. It should be noted that workers such as Carp et al.[4] and Cowchock et al.[3] based their observations on the comparisons between immunized and non-immunized women. This method ignores any psychological effect of treatment, be it with lymphocytes or placebo.

In our series, a success rate of 76% in the non-immunized controlled group was higher than that reported in most studies, and merely emphasizes the variation that can be obtained in various studies in relation to untreated patients. Studies reporting a placebo effect of less than 60% are clearly so different from most other studies that the adequacy of blinding of such studies must be seriously questioned.

Other factors that have been reported to be associated with improved results of white cell immunization include restricting the procedure to primary aborters and only those with recurrent missed abortions (i.e., exclude the live aborter), or giving white cells from a much larger blood sample obtained from the partner. These factors would seem to have little relevance in our study, as the success rate in the non-immunized control subjects was so high.

This study does not allow us to draw any conclusions as to what factors are important in predicting the success of the subsequent pregnancy. An analysis of the combined data from this controlled trial as well as from a previously uncontrolled trial (unpublished observations) would indicate that factors relating to subfertility correlate best with subsequent performance, as we suggested earlier.[11] This seems to be supported by some recent data reported by Peters and Coulam.[12]



Paired sequential trial chart for: Θ=0.85, 2=0.05

I- β=0.95, N=27, ASN=13.4

Fig. 1. Paired sequential trial chart. Each point on the curve represents the result from a pair of habitual aborters given partner's cells or normal saline. If both had the same result (i.e., normal pregnancy or abortion) then the line was projected horizontally. If there was success (pregnancy with partner's cells and failure with normal saline) the line was projected upwards; otherwise, the line was projected downwards. Touching the upper or lower boundary would indicate preference for immunization with partner's cells or saline respectively. Touching the right boundary, as in this case, indicates that the result of treatment was unlikely to be significantly different at the 0.05 level.[23]

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EXHIBIT D

Mononuclear-cell immunisation in prevention of recurrent miscarriages: a randomised trial

Carole Ober, Theodore Karrison, Randall R Odem, Randall B Barnes, D Ware Branch, Mary D Stephenson, Beverly Baron, Mary Ann Walker, James R Scott, James F Schreiber

Summary

Background Couples with unexplained recurrent miscarriage may have an alloimmune abnormality that prevents the mother from developing immune responses essential for the survival of the genetically foreign conceptus. Immunisation with paternal mononuclear cells is used as a treatment for such alloimmune-mediated pregnancy losses. However, the published results on this treatment are conflicting. In this study (the Recurrent Miscarriage (REMIS) Study), we investigated whether paternal mononuclear cell immunisation improves the rate of successful pregnancies.

Methods Women who had had three or more spontaneous abortions of unknown cause were enrolled in a double-blind, multicentre, randomised clinical trial. 91 were assigned immunisation with paternal mononuclear cells (treatment) and 92 immunisation with sterile saline (control). The primary outcomes were the inability to achieve pregnancy within 12 months of randomisation, or a pregnancy which terminated before 28 weeks of gestation (failure), and pregnancy of 28 or more weeks of gestation (success). Two analyses were done: one included all women (intention to treat), and the other included only those who became pregnant.

Findings Two women in each group received no treatment, and eight (three treatment, five control) were censored after an interim analysis. In the analysis of all randomised women who completed the trial, the success rate was 91/86 (36%) in the treatment group and 41/85 (48%) in the control group (odds ratio 0·60 [95% CI 0·33–1·12], p=0·108). In the analysis of pregnant women only, the corresponding success rates were 31/98 (46%) and 41/83 (65%; odds ratio 0·45 [0·22–0·91], p=0·026). The results were unchanged after adjustment for maternal age, number of previous miscarriages, and whether or not the couple had had a previous viable pregnancy. Similar results were obtained in a subgroup analysis of 133 couples with no previous livebirth.

Interpretation Immunisation with paternal mononuclear cells does not improve pregnancy outcome in women with unexplained recurrent miscarriage. This therapy should not be offered as a treatment for pregnancy loss.

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Introduction

About 15% of clinically recognised pregnancies are spontaneously aborted;[1] thus, miscarriage is the most common complication of human pregnancy. Although most miscarriages are sporadic, recurrent miscarriage (three or more spontaneous abortions) occurs in 0·5–1·0% of couples.[2] In most women who experience recurrent miscarriage, no cause can be identified.[3,4] Alloimmune mechanisms that prevent mothers from developing immunological responses essential for the survival of the semiallogeneic pregnancy have been proposed as the cause of some or all of these losses.[4] On the basis of animal models of abortion and studies of human organ transplant survival, immunisation with paternal white cells was proposed as a treatment for alloimmune-mediated pregnancy loss.[5,6] This immunotherapy is offered by many medical centres in the USA and elsewhere, although its efficacy remains controversial.[7,8] Published trials and meta-analyses of published and unpublished studies have yielded conflicting results, indicating the need for large randomised trials.[9] The purpose of this multicentre, randomised, double-blind trial, the Recurrent Miscarriage Study (REMIS), was to assess the efficacy of immunisation with paternal mononuclear cells as a treatment for unexplained recurrent miscarriage.

Methods

Patients

Patients were recruited at six centres between July, 1992, and December, 1997: University of Chicago, Chicago, IL; Washington University School of Medicine, St Louis, MO; University of Utah, Salt Lake City, UT; University of Pittsburgh, Pittsburgh, PA; Los Olyvos Women's Center, Del Gatos, CA, USA; and University of British Columbia, Vancouver, British Columbia, Canada. All study centres had approval from their institutional review boards, and informed consent was obtained from all patients. Since patients were recruited from a wide referral base, the number screened, and the number ineligible was not known.

The eligibility criteria were: three or more previous miscarriages (not necessarily consecutive) that were not of chromosomally abnormal fetuses or ectopic pregnancies; no more than one liveborn child with the current partner; age 40 years or younger at the time of recruitment; not pregnant at the time of immunisation; no anti-HLA antibodies measured by a microcytotoxicity assay; no contraindications for immunisation with paternal mononuclear cells; and no identifiable cause for the previous miscarriages. The latter criterion was confirmed by means of cytogenetic studies in both parents; luteal-phase serial progesterone measurements or in-phase endometrium;[10] concentrations of thyroxine in serum; intrauterine contour assessed by hysterosalpingography, sonohysterography, or hysteroscopy; and assays of antibodies to cardiolipin, which were measured in a single laboratory,[11] and lupus anticoagulant assayed by normal clotting times (within 2 SD of the mean) in sensitive, phospholipid-dependent clotting assays. Most centres used a sensitive partial thromboplastin time, and some centres used the dilute Russell's viper venom time.

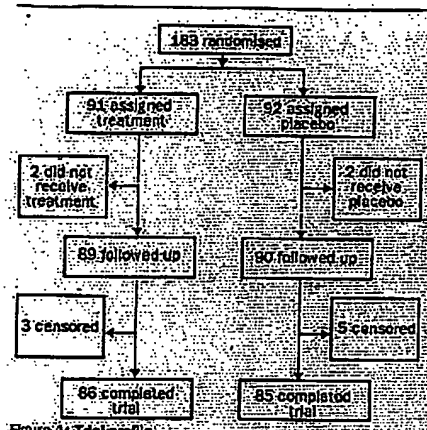


Figure 1: Trial profile

Design and procedures

Patients were randomly assigned at each centre to either a treatment or control group. The randomisation was stratified by clinical centre with permuted blocks of size eight and ten.[8] Women assigned to the treatment group were immunised with paternal mononuclear cells, and those assigned to the control group were injected with an equivalent volume of sterile saline. Opaque, sequentially numbered, sealed envelopes, prepared by the study biostatistician were kept at the Blood Bank (USA) or Transfusion Medicine Center (Canada) of each centre, and were opened by laboratory personnel when the male partner donated blood (1 day before immunisation). The donated blood was discarded if the couple was assigned to the control group. Syringes for immunisation were prepared by blood-bank personnel and given to the REMIS nurse coordinator, who administered the injections. Syringes and tubing were covered with opaque tape so that cloudy cell solutions could not be distinguished from clear saline. Thus neither the patients nor the study personnel who had contact with the patients were aware of the treatment allocation.

Mononuclear cells were removed from one unit of whole blood with the use of a Ficoll gradient, and were stored overnight at 1–6°C. The next day, the cell preparation (containing about 200 million lymphocytes in 5 mL normal saline) was placed in syringes. 3 mL were administered intravenously and 0·5 mL volumes were injected in two subcutaneous and two intradermal sites on the forearm.[9] For women assigned to the control group, 5 mL saline was divided into syringes and administered in an identical manner. All women were immunised during the first 2 weeks of their menstrual cycle after a negative pregnancy test. They were monitored for 1 h after immunisation, and any reactions to the immunisation, as well as temperature, blood pressure, and pulse rate, were recorded. Antibodies to paternal HLA were measured 2 or more weeks after immunisation, but the results were not revealed to the investigators. All patients were contacted by telephone every 3 months. Those who did not become pregnant within 6 months were reimmunised with the same treatment as they received initially, following the same protocol.

Pregnancy was diagnosed 1–5 days after a missed period—ie, during the third week after ovulation (5 weeks of gestation). Weekly visits throughout the first trimester of pregnancy were scheduled after pregnancy was confirmed; when visits were not possible, the patients were connected by telephone. Supportive therapy was provided during the weekly visits by the patient's physician or REMIS nurse coordinator; this therapy included psychological support and ultrasonographic examinations.[3]

After the first trimester, obstetrical care was assumed by the patient's own physician. The nurse coordinators continued to contact each woman monthly during the remainder of the pregnancy. For women who progressed normally, umbilical cord blood and placental samples were collected at delivery for genetic studies. If a miscarriage occurred, every effort was made to collect the products of conception for cytogenetic and genetic studies. Pregnancy losses were considered to be pre-embryonic if they occurred before the documentation of fetal cardiac activity, embryonic if they occurred after establishment of fetal cardiac activity but before 10 weeks of gestation, and fetal if they occurred after establishment of fetal cardiac activity and after at least 10 weeks of gestation.[10] No other concomitant therapies for recurrent miscarriage were used.

	Treatment group (n=89)	Control group (n=90)
Age (years)*	32·7 (4·5; 23–41)†	32·7 (4·6; 23–40)
Race		
White	69 (93%)	78 (87%)
Other	6 (7%)	12 (13%)
Pregnancy history		
Number of previous pregnancies*	4·0 (2·1; 2–10)	4·0 (2·4; 2–9)
Number of previous miscarriages*	4·0 (1·8; 2–13)	4·0 (1·4; 2–9)
Number of women with previous preterm deliveries	28 (33%)	17 (20%)
Number of women with previous ectopic pregnancies	9 (10%)	12 (13%)
Number of women with a previous chromosomally abnormal miscarried fetus	5 (6%)	7 (8%)

*Mean (SD; range); one woman was excluded at age 40 but underwent delivery for data analysis.
†Age at delivery.

Table 1: Demographic variables and pregnancy history by treatment group

Statistical analysis

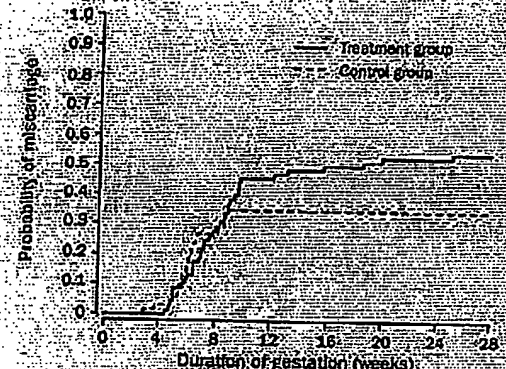
The primary analysis was by intention to treat carried out on all randomised patients. Success was defined as a pregnancy that continued to at least 28 weeks of gestation, following the protocol of Mowbray and colleagues.[3] Treatment failures were women who did not become pregnant within 12 months of randomisation, and women who experienced a pregnancy loss before 28 weeks of gestation. A secondary analysis was done including only women who became pregnant within 12 months of randomisation, with a failure defined as a pregnancy loss before 28 weeks of gestation. A subgroup analysis limited to women with no previous liveborns was also done.

Baseline differences between the treatment and control groups were analysed by means of Student's t test for continuous variables, and by χ^2 or Fisher's exact test for categorical variables. Success rates were compared by logistic regression analysis to derive odds ratios, both unadjusted and adjusted for three factors known to be associated with miscarriage (ie, maternal age, number of previous miscarriages, and whether or not the patient had a previous liveborn child). The distributions of the number of months to a positive pregnancy test after randomisation, and the number of weeks to a miscarriage after pregnancy diagnosis were estimated by the Kaplan-Meier method, and were compared between the two groups by the log-rank test.

The study was designed to detect an increase in the rate of livebirths among pregnant women (specifically a gestation of 28 weeks or more) from 60% in the control group to 80% in the treated group, assuming equal pregnancy rates of 60% in both groups (ie, a difference in success rates of 12%). A target sample size of 262 women per group was necessary to have 80% power

Pregnancy outcome	Treatment group	Control group
All randomised patients		
Total	84	85
Success	51 (61%)	41 (48%)
Failure	33 (39%)	44 (52%)
All pregnant patients		
Total	68	80
Success	51 (75%)	41 (51%)
Failure	17 (25%)	39 (49%)

Table 2: Study outcomes by treatment group



Number at risk								
Treatment	88	88	49	37	34	33	32	32
Control	93	91	66	43	41	33	31	31

Figure 2: Time from pregnancy to miscarriage among patients immunised with paternal mononuclear cells (treatment—solid line) and patients immunised with sterile saline (control—dotted line)

to detect a difference of this magnitude, with a two-sided significance test at $\alpha=0·05$. Interim analyses were planned after every 50 outcomes, with an O'Brien-Fleming monitoring boundary.[7] However, the sample size was smaller than planned when the last participant was randomised in December, 1997, and only three interim analyses and one final analysis were done. All results were reviewed by an independent data and safety-monitoring committee. After the third interim analysis in February, 1998, the committee recommended that no further 6-month reimmunisations be given. The reason for this decision was that the miscarriage rate in the treatment group was higher than that in controls. Consequently, three women in the treatment group and five in the control group who did not become pregnant at 6 months were not reimmunised. For the intention-to-treat analysis, outcomes from these eight patients were considered indeterminate and were excluded from the group comparisons. In the analysis of the distribution of the number of months to a positive pregnancy test, observations from these patients were "censored" at 6 months—ie, no follow-up information beyond 6 months was used in the calculation.

Results

183 women were randomly assigned treatment or placebo (figure 1). Two in each group were disqualified before immunisation; results of screening indicated that one was pregnant and one had a blood-group incompatibility; two other couples decided not to take part after randomisation (but before immunisation), one because the husband was positive for cytomegalovirus antibodies, and one for personal reasons. 131 (73·2%) of the 179 women became pregnant within 12 months of randomisation, 40 (22·3%) did not, and for eight the result was indeterminate. Among the 131 pregnant women, 72 (55%) delivered and 59 (45%) had miscarriages. Among the 59 pregnancy failures, five were ectopic, 31 were pre-embryonic, 17 were embryonic, and six were fetal.

The distribution of demographic and pregnancy history variables in the treatment and control groups is shown in table 1. The groups were similar except that a higher proportion of women in the treatment group had had a previous livebirth (p=0·054). This variable, along with maternal age, and number of previous losses, were included as covariates in subsequent analysis.

In the intention-to-treat analysis, the success rate was 36% in the treatment group and 48% in the control group (table 2; odds ratio 0·60 [95% CI 0·33–1·12], p=0·108). The corresponding analysis adjusted for maternal age, number of previous miscarriages, and previous livebirth gave a similar odds ratio (0·54 [0·28–1·02], p=0·056). None of the covariate effects reached statistical significance, although a previous livebirth was associated with greater odds of success, of marginal significance (2·05 [0·96–4·35], p=0·062). Kaplan-Meier estimated pregnancy rates did not differ significantly between the groups: 78% in the treatment group and 72% in the control group (log-rank p=0·232).

In the analyses that included pregnant women only, the success rate was 46% in the treatment group and 65% in the control group (odds ratio 0·45 [0·22–0·91], p=0·025). The corresponding analysis adjusted for covariates again gave a similar odds ratio (0·40 [0·19–0·84], p=0·015). In this analysis, the number of previous pregnancy losses had a significant effect on success rate (odds ratio 0·75 per additional loss [0·58–0·99], p=0·040). The treatment effects were also similar among the participating centres (data not shown).

Analyses were repeated for couples with primary recurrent miscarriage—ie, couples without a previous livebirth. The results again favoured the control group, with success rates in the intention-to-treat analysis of 18/59 (30%) in the treated group and 32/70 (46%) in the controls (odds ratio 0·52 [0·25–1·08], adjusted p=0·082). Among pregnant patients, success rates were 18/46 (39%) and 32/51 (63%) in the treatment and control groups, respectively (0·37 [0·16–0·86], adjusted p=0·021). In the treatment group, 26% of patients developed HLA antibodies after immunisation. There was no evident association between success rate and HLA-antibody status (31% and 30% for patients with and without HLA antibodies after immunisation, respectively; p=1·0).

The distributions of time from pregnancy to miscarriage in the treatment and control groups are shown in figure 2. The mean duration of gestation at the time of miscarriage was 8·9 weeks (SD 4·5) and 6·2 weeks (1·5) in the treatment and control groups, respectively (p=0·002). The duration of gestation at which miscarriage occurred was before 10 weeks in all patients in the control group, whereas six (16%) of 37

	Developmental stage			
	Ectopic	Pre-embryonic	Embryonic	Fetal
Treatment group (n=88 pregnancies)				
Total failed pregnancies	3 (13%)	18 (28%)	12 (18%)	6 (9%)
Number for which cytogenetic studies were available	0	4	9	4
Number of normal karyotypes (46,XX/46,XY)	..	2/0	0/4	3/1
Abnormal karyotypes	..	47,XX+20	45,X	..
	..	47,XX+16	45,X	..
	..	..	47,XX+16	..
	..	..	47,XX+16	..
	..	..	69,XXX	..
Control group (n=83 pregnancies)				
Total failed pregnancies	4 (16%)	13 (21%)	5 (8%)	0
Number for which cytogenetic studies were available	1	1	2	..
Number of normal karyotypes (46,XX/46,XY)	0/1	1/0	2/0	..
Abnormal karyotypes	..	..	..	..

Table 3: Developmental stages and abnormalities in failed pregnancies

losses in the treatment group occurred after 10 weeks of gestation. The timing of pregnancy loss by stage of development is shown in table 3. Ectopic, pre-embryonic, and embryonic loss rates did not differ significantly between the treatment and control groups (p=0·320, 0·564, and 0·162, respectively), but the rate of fetal loss was significantly greater in the treatment group (p=0·036).

Chromosome studies in the products of conception were successful for 21 of the 59 abortuses. Seven (33%) of the 21 abortuses on which cytogenetic studies were done had abnormal karyotypes; all were in the treatment group (table 3). Four additional fetuses in the treatment group had abnormalities: two had a cystic hygroma, one had an omphalocele, a tracheo-oesophageal fistula, duodenal atresia, and a single umbilical artery; and one had an abnormal triple screen (α-fetoprotein, unconjugated oestriol, and human chorionic gonadotropin).

There were no pregnancy losses or fetal deaths after 28 weeks of gestation. There were no differences with respect to delivery statistics between infants born to mothers in the treatment group (31 liveborn) and those born to mothers in the control group (41 liveborn). The mean gestational ages at delivery were 39·2 weeks (SD 1·7; range 35·6–44·4) in the treatment group and 39·4 weeks (1·3; 36·4–41·0) in the control group (p=0·698). The mean birthweights in the two groups were 3395 g (623; 2241–4592) and 3353 g (491; 2381–4479), respectively (p=0·755). Sex ratio (M/F) was 0·82 in the treatment group and 0·86 in the control group (p=1·0).

Discussion

In the REMIS study, the pregnancy success rate was higher in the control group than in patients immunised with paternal mononuclear cells, irrespective of whether all randomised patients or only patients achieving a pregnancy were considered, or whether or not patients with previous liveborn children were excluded. In addition, outcomes were similar among patients in the treatment group irrespective of whether they developed HLA antibodies after immunisation. Thus, we found no evidence of benefit from immunisation with paternal mononuclear cells for prevention of recurrent miscarriage. Furthermore, higher rates of pregnancy loss among patients immunised with paternal cells than those immunised with saline suggests that immunotherapy with paternal mononuclear cells may increase the rate of clinically recognised pregnancy losses.

The higher rate of miscarriage in the treatment group was associated with losses occurring later in gestation. Indeed, all losses occurring after 9 weeks of gestation were in the treatment group (figure 2). All chromosomal and other abnormalities also occurred in the treatment group. However, this finding probably reflects the greater chance of identifying fetal tissues in the products of conception later in gestation, and the larger number of later pregnancy losses in the treatment group. For example, in this study, chromosome analyses were successful in five (16%) of 31 pre-embryos, 11 (65%) of 17 embryos, and four (67%) of six fetuses (table 3). Extrapolation of epidemiological studies of chromosome abnormalities in abortuses[11] suggests that a significant proportion of the earlier losses were chromosomally abnormal. However, without knowledge of the chromosomal status of all abortuses we cannot confine our analyses to chromosomally normal pregnancies. Nevertheless, given the almost identical maternal age distribution among the treatment groups (table 1), the occurrence of chromosome abnormalities should have been randomly distributed among the groups.

The results of this study differ from those of the smaller clinical trial by Mowbray and colleagues. Although we followed an almost identical protocol, there were some differences between the studies. First, the previous study used maternal mononuclear cells from 10 mL blood as the control treatment, whereas we used sterile saline. Because of the lower than expected pregnancy success rate in the control group in that study (37%), we chose to use saline, as in the trial of Cauchi and colleagues. Second, Mowbray and colleagues' trial did not provide first-trimester supportive therapy. The use of saline as a placebo and the provision of supportive therapy in the first trimester could account for the greater success rate in our control group than in Mowbray and colleagues' study (65 *vs* 37%, respectively, among pregnant women), although the success rate in our control group is similar to success rates reported in epidemiological and cohort studies of women with recurrent miscarriage. However, these differences in our protocols are unlikely to account for the disparity in success rates in the treatment groups between the two studies (46 *vs* 77%, respectively, among pregnant patients). On the other hand, differences in experimental design and analysis could explain some of the differences between our studies. Mowbray and colleagues used a fully sequential design (stopping early) and did not analyse by intention to treat, as we did, which may have biased the outcome if either pregnancy rates or rates of preclinical pregnancy losses differed between the two groups. Also, when 18 more patients in Mowbray and colleagues' study (analysed by Jeng and colleagues) were followed up after their trial had stopped, the treatment effect was lower, and the differences between treated patients and controls were not significant.

In one meta-analysis, which included published and unpublished data from the study of Mowbray and colleagues and from several additional randomised trials, a small but significant effect in favour of leucocyte immunotherapy was found. In this analysis, the success rates in treated women ranged from 62% to 77% (pooled sample, 68·4%). Results were reported on a relative-risk scale—ie, as the ratio of livebirth rates in treated and control groups. In that paper, two analysis teams worked independently and arrived at estimated ratios of 1·16 (95% CI 1·01–1·34) and 1·21 (1·04–1·37), respectively. In a 1996 meta-analysis including updated data from these trials, the effect of immunotherapy did not reach significance (livebirth rate ratio 1·12 [0·97–1·31]). If the results from our trial are added to these data, the estimated livebirth rate ratio falls to 1·04 (0·91–1·20). A test for heterogeneity of the results across the trials was not significant (p=0·320).

In this study, immunisation with paternal mononuclear cells did not improve pregnancy outcome in women with recurrent miscarriage. Despite a history of unexplained recurrent miscarriage, nearly 65% of control patients who became pregnant had a successful pregnancy. We found a higher rate of miscarriage and a greater gestational age at the time of loss in immunised women who became pregnant. Because of the lack of benefit, we recommend against this intervention as a treatment for unexplained recurrent miscarriage.

Contributors

Carole Ober, Theodore Karrison, Randall Odem, Randall Barnes, Ware Branch, Mary Stephenson, Beverly Baron, James Scott, and James Schreiber were involved in the study design, execution, data analysis, and writing of this work. Mary Ann Walker coordinated the day-to-day activities, collected data on patients, and contributed to the preparation of the paper.

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